

THE KINETICS OF TRYPTIC ACTION

BY

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The Kinetics of Tryptic Action.

By W. M. BAYLISS, F.R.S.

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The research, of which the present paper may be regarded as a preliminary account, was originally undertaken with a view of throwing light on the nature of the various modes of action of the pancreatic juice. It was found necessary to investigate, first of all, the properties of the comparatively simple commercial preparations of trypsin, and this part of the subject has shown itself to be of so complex a character that, hitherto, very little work has been done with the natural juice.

There is not much to be said with regard to previous work on the physical chemistry of trypsin. The most important is that of Victor Henri¹ which has been published during the progress of the present investigation. This, as well as other papers bearing on the subject, will be most profitably discussed when occasion arises.

The experimental investigation of trypsin from the physico-chemical point of view is involved in many difficulties. I may mention the facts of its

¹ C. R. Soc. de Biologie, 1903 and 1904.

instability, especially in solutions free from proteid or products of proteid disintegration, and also that its action on proteids takes place in stages, albumoses and peptones, for example, being further split into amino-acids, etc., as shown by the gradual disappearance of the biuret reaction. Further, the powerful action of the products of the reaction on the rate of change introduces an additional complication, which is increased by the fact that these various products do not appear to be equally active in this respect. For this latter reason I have devoted some attention to the action of trypsin on the glycin-base (polypeptide of Emil Fischer) described by Curtius¹ and which, as shown by Schwarzschild², is split into glycin and alcohol, so that the concentration of the substrate³ and of the products of its change are known at all stages of the reaction.

In the hope of elucidating the question of the relative concentration of the various products in different stages of the reaction, I have thought it useful to follow the rate of change by various methods simultaneously, as for example, electrical conductivity, internal friction, refractive index, etc., as well as by chemical methods such as the biuret and Millon reactions, the inorganic phosphates, the total nitrogen (by Kjeldahl) in filtrates after precipitation in various ways, and so on.

GENERAL METHOD.

I have made most use of the electrical conductivity method as introduced by Victor Henri into the study of enzymes. This method has many advantages, the readings are made rapidly and with considerable accuracy, so that it is easy to obtain a large number of readings at short intervals of time, a fact of much importance in the early stages of the reaction. It is possible also to perform a great variety of experiments under various conditions, which it would be impossible to do owing to the time involved, by means of a chemical method. A further advantage is that the vessel in which the reaction is proceeding is kept in the thermostat completely undisturbed during the progress of the experiment. In certain cases, as when we have at our disposal only small amounts of material, for example, pure preparations of various products of tryptic or peptic action, the small quantity of fluid necessary is of importance.

¹ Ber. d. Deutsch. Chem. Ges. 16, 1, p. 753.

² Hofmeister's Beiträge, IV, p. 162.

³ I must make use of this word, for want of a better, to express the body being acted upon by the enzyme; "Hydrolyte," used by some authors, is more limited in its application and does not include, for instance, bodies oxidized under the action of oxydases.

The electrical conductivity of a tryptic digestion shows, as a rule, a continuous increase during the progress of the reaction. Whether this is due to an actual increase of electrolytes or to some "viscosity" effect is not yet clear, although I have some data to bring forward in reference to the matter. This, of course, does not militate against the value of the method as an extremely convenient one for following the course of the reaction. The method used for measuring the electrical conductivity was that of Kohlrausch with rapidly alternating currents and telephone¹. The vessels were those of Henry as supplied by Fritz Köhler of Leipzig². These vessels can readily be sterilized either in steam or hot air, they are stoppered so that no evaporation takes place, and the electrodes are vertical at the sides so that any solid particles fall to the bottom and fluid can be pipetted out without danger of displacing the electrodes. The electrodes were platinized, a matter of great importance in respect of the sharpness of the reading, and replatinized at intervals. The resistance-capacity of all vessels used was determined by $\frac{n}{10}$ KCl at various times in order to detect accidental changes in the position of the electrodes; the absolute capacity in most experiments, however, was not of great importance, but the relative capacities must be accurately known.

The thermostat used was of Ostwald's pattern, with toluol regulator. Here I may mention that I have found it advisable to fill the reservoir to only about two-thirds with toluol in order to avoid the slow creeping of the toluol round the bend of the tube as referred to by Ostwald³. Although the sensitiveness of the regulator is somewhat diminished by this proceeding, it is quite sensitive enough, keeping the temperature constant to 0.1° .

As a convenient unit of conductivity I use the reciprocal megohm or "gemmho" as introduced by Waller. In these units the specific conductivity of $\frac{n}{10}$ KCl is 11,190 gemmhos, 2.84% caseinogen (as Na-salt) 1160⁴, 5% gelatin (gold label) 125, all at the temperature of 18° .

Other methods used in special cases will be described later under the particular experiments in question.

Various proteids were used as substrate, but chiefly caseinogen and gelatin. The caseinogen prepared by Hammersten's method was obtained from Gröbler and Merck. It was brought into solution by the appropriate quantity of sodium hydrate⁵, the reaction then being acid to phenol-phthalein,

¹ Kohlrausch and Holborn. *Leitvermögen der Elektrolyte*, 1898.

² See his "Preis-Verzeichnis," 1903, p. 66, fig. 121.

³ Ostwald-Luther. *Physik.-chem. Messungen*, 1902, p. 92.

⁴ Laqueur and Sackur. *Hofmeister's Beiträge*, III., p. 199.

⁵ Laqueur and Sackur, l. c.

and alkaline to litmus. It was usually centrifuged in order to separate a certain amount of insoluble body (perhaps the "Natriumkaseid" of Laqueur and Sackur). The calcium and ammonium salts of caseinogen were also sometimes used.

Gelatin (Coignet's gold label) was employed in watery solution.

Caseinogen has the useful property of being rapidly attacked by trypsin, so that an experiment can be completed in a few hours, so far indeed as requisite for many purposes.

The trypsin was obtained from Grüber, Merck, and the Company "Rhenania" of Aachen. These preparations did not dissolve completely in distilled water, but it was found that the solutions were not less active when filtered, so that the insoluble body was not the enzyme.

Pancreatic juice obtained by injection of "secretin," activated by enterokinase or unactivated, and glycerin extracts of pancreas were occasionally employed.

I. The Velocity of Reaction.

1. General features of the action of trypsin on caseinogen and gelatin.

A solution containing 8% sodium caseinogenate was prepared as described above and to 6 c. c. there was added 2 c. c. of $\frac{n}{2}$ ammonia. It

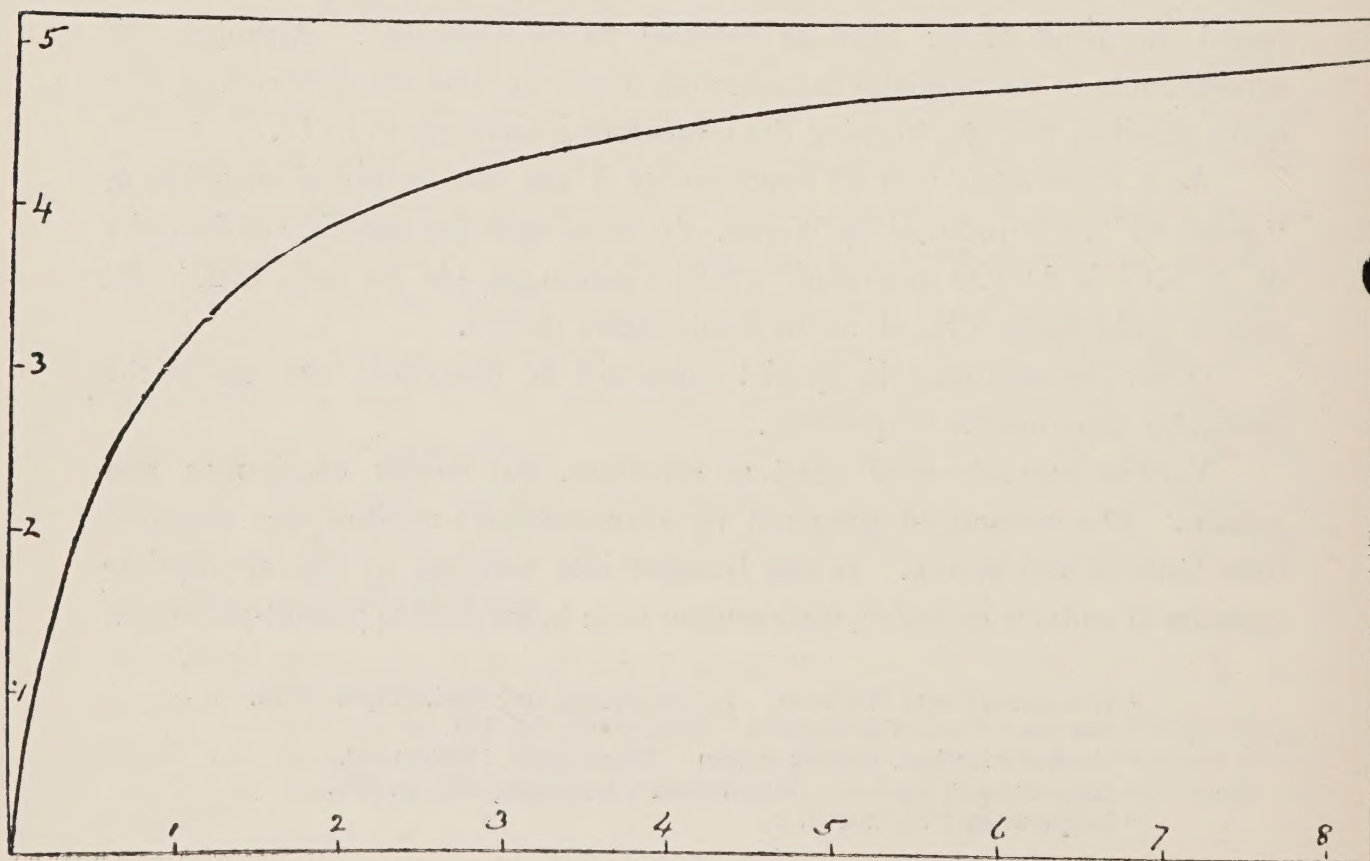


Fig 1

was then placed in the thermostat at 39° with a few drops of toluol for some hours. A 2% filtered solution of trypsin was then warmed in a test-tube in the thermostat until a thermometer immersed in it showed that it had attained the thermostat temperature. 2 c. c. of the warmed trypsin solution were added by means of a warmed pipette to the caseinogen solution, and two minutes later the conductivity readings were commenced. From the values obtained the curve fig. 1 was constructed. The ordinates represent changes of conductivity in gemmhos $\times 100$, and the abscissae time in hours.

The general form of the curve will be seen to resemble that of a logarithmic curve. The steepness of the curve, which represents the rate of change at any given period of time, steadily decreases as the reaction progresses, and this means that the velocity of reaction at any time depends on events which have taken place in previous stages of the reaction.

Now if the trypsin reaction obeys the law of mass-action the rate of change at any moment will be proportional to the concentration of the caseinogen at this moment according to the equation.

$$-\frac{dC}{dt} = KC^n$$

where C is the concentration at the time t , k is the velocity constant and n a number dependent on the number of molecules taking part in the reaction.

As a first approximation we will assume that the trypsin reaction is unimolecular, in which case $n = 1$.

Since the action of trypsin does not come to an end in a period of time sufficiently short for experimental purposes, we must take for the purpose of calculating the velocity constant, the following form of the integral of the above equation, viz. :

$$K = \frac{1}{t_2 - t_1} \log. \text{ nat. } \left(\frac{C_1}{C_2} \right)$$

in which C_1 and C_2 are the concentrations, at times t_1 and t_2 respectively, and it is not necessary to know the initial or final concentration.

From the data employed in the construction of curve, figure 1, it is possible to calculate the velocity constants at various times, taking for the purpose the change in conductivity as being proportional to the change in concentration of the substrate. For the present purpose, as the absolute values of the constants are not essential, I have used ordinary logarithms instead of natural logarithms. At the beginning of the reaction, where the

change is rapid, the constants are taken for each 10 minute interval, later on for 20 minute intervals.

1st 10 minutes	$k = 0.0079$
2nd "	0.0046
3rd "	0.0032
4th "	0.0022
5th "	0.0016
7th "	0.0009
9th "	0.00065 and so on.

It will be seen that there is a continuous and rapid fall in the value of the velocity constant when calculated by the simple unimolecular equation. The same fact may be represented graphically by plotting out a logarithmic curve on the basis of the velocity constant at the commencement of the reaction and comparing it with fig. 1. The true logarithmic curve will be seen to rise higher than the trypsin curve and to diverge from it more and more as the reaction proceeds. Or we may compare the curve of inversion of cane-sugar by acid, which at certain concentrations is logarithmic, with our trypsin curve.

How is this phenomenon to be explained? There are probably several factors at work, but two in particular suggest themselves at once. Enzymes, being colloids, are, like other colloids, unstable in solution, and trypsin is known to be particularly unstable, so that, during the course of a digestion, the trypsin content may be steadily diminishing, which would account for the slowing of the rate of change. But the second factor is, as we shall show later, the more potent one. I refer to the effect of the products of the reaction, which have a markedly retarding action on the progress of the change. This fact is well-known to be more or less general in the case of enzyme-action, but has been particularly worked out by Tammann¹ in the case of emulsin.

There are possibly other subsidiary factors concerned, such as the change of the physical condition of the solution, shown by the diminution of the internal friction; these will be referred to later.

Before we proceed to analyse these various factors more minutely it will be well to see if there is any relation between the concentration of the substrate and the velocity of the reaction, apart from the retarding factors.

2. Effect of concentration of substrate.

Victor Henri finds² that, at all events during the first hour of the action of trypsin on gelatin, the changes of conductivity follow the unimolecular law;

¹ Zeitsch. f. physik. Chemie, XVIII., p. 427.

² Archivio di Fisiologia, I., p. 307.

the calculations are based, however, on a formula which assumes the application of the logarithmic law to the particular case in question. This formula is for the purpose of calculating the initial concentration. I do not find that it applies to my results, since the values obtained for the initial concentration vary according to the stage of the reaction from which the data are taken.

In order to observe how the initial concentration of the substrate affects the rate of change, I took a series with varying concentrations of caseinogen but of the same trypsin content. To eliminate as far as possible the retardation I give in the table the amount of conductivity change in the first 20 minutes :

Caseinogen Content.	Conductivity change in 20'.
4 ^o / _o	385 gemmhos.
3.2 ^o / _o	395 "
2 ^o / _o	200 "
1.6 ^o / _o	182 "
0.8 ^o / _o	110 "
0.4 ^o / _o	10 "

In this case we see that up to 3.2% there is an increase in the rate of change, but that it does not increase as fast as the concentration does, and that at 4% it is less than at 3.2%. In another experiment with more concentrated caseinogen and less trypsin I found the rate of change equal for 8%, 5% and 4% and less for 10%.

This phenomenon is more marked in the case of gelatin :

Concentration of gelatin.	Conductivity change in 25.'
10 ^o / _o	130
8 ^o / _o	170
4 ^o / _o	240
2 ^o / _o	280

These results correspond exactly with those of Victor Henri in the case of invertase¹.

The probable explanation of the lessened rate of change in solutions of substrate above a certain concentration in relation to the amount of enzyme present is to be found in the mode of action of trypsin. The most generally accepted view of enzyme-action in general is that there is a transitory, intermediate, combination between the substrate and the enzyme, which then splits up with production of products of decomposition of the substrate and setting-free of the enzyme. If, therefore, there is present more than that amount of substrate with which the trypsin can combine, the rate of change will not be increased, and the actual diminution observed when the con-

¹ Archivio di Fisiologia, loc. cit., p. 313.

centration is considerable may be due to the large increase of internal friction. The results of Medwedew¹, with respect to the oxydase of the liver, are of interest in this connection.

Arrhenius² has called attention to the close relationship between the electrical conductivity as diminished by non-electrolytes and the internal friction of the solution. If, however, colloidal solutions are suspensions of ultra-microscopic solid particles it might be supposed that the addition of colloid to an electrolyte would not affect its conductivity. This, in fact, appears to be the case with some colloids, but not all. I took solutions of gelatin varying from 5 to 0.5 per cent., the gelatin having been soaked in several changes of distilled water, and, for comparison, the last change of water in which the gelatin had been soaked, they were placed in the thermostat at 40° and the same amount (1 c. c.) of $\frac{n}{10}$ KCl added to each. The increase of conductivity was the same in all, including the water. This non-effect of gelatin was not due to its being in the "gel" state, since it was melted at 40°. Caseinogen, on the contrary, has an effect similar to that of non-colloidal non-electrolytes, such as sugar, though less marked. This question needs further investigation, as well as that of the influence of non-electrolytes, especially colloids, on the velocity of reaction in general.³

The result we arrive at is, then, that within the limits usually taken in the present series of experiments there is a proportionality between the concentration of the substrate and the velocity of the reaction, but not a direct, linear, proportionality. So far we may say that trypsin action obeys the law of mass-action, in that its rate of change is some function of the concentration of the substrate.

It is of interest to note that at the end of the curve, figure 1, there was still present a small amount of unattacked caseinogen, that is, precipitable by acetic acid.

This curve being compounded of a logarithmic curve, due to the diminution of concentration of the substrate, and retarding influences due to the increase of products of the reaction and possibly the disappearance of the enzyme, it would be expected that if we could keep these factors constant the curve would become a straight line. I have been able to obtain this very approximately by hanging a small inverted glass bell, filled with solidified jelly on one arm of a delicate balance, while a straw lever attached to the

¹ Pflüger's Archiv. Bd. 103, p. 403.

² Zeitsch. f. physik. Chemie. Bd. 9, p. 487.

³ As to non-effect of certain colloids (gelatin, agar-agar and colloidal silica) see Reformatsky (Zeits. f. physik. Chemie. Bd. 7, p. 34) and Levi (Chem. Centralblatt, 1900, II., p. 658).

other arm traced on a slowly-moving smoked paper; the bell was immersed in a considerable volume of trypsin solution and allowed to digest at room temperature. The straw lever slowly fell as the gelatin was dissolved and traced a straight line on the paper. I hope to perfect this apparatus and some interesting results may be expected.

It might be possible, also, to eliminate the effect of concentration of substrate by taking suspensions of solid particles. I have tried this with finely-chopped fibrin. The result, however, is difficult to interpret. The fibrin was apparently rapidly converted into a soluble form, since the solution, after disappearance of all solid particles, coagulated on boiling. This soluble proteid was then further split up by the enzyme.

3. Destruction of Enzyme.

We now turn our attention to the first of the modifying factors mentioned above. The rapid disappearance of activity at 38° in a fairly pure solution of trypsin was shown by Vernon¹; he also pointed out the protection afforded by the presence of proteid. The present author in conjunction with E. H. Starling found that Witte's "peptone" has a similar preservative effect. It seems probable, then, that in digestion mixtures, if not carried on too long, the destruction of trypsin may be inappreciable. Victor Henri² has pointed out a method of testing this, which I have applied in the following way. A number of conductivity vessels, each containing 5 c. c. of a 5% solution of caseinogen, stood ready in the thermostat. A large flask of caseinogen solution with trypsin also stood in the same thermostat; at various intervals samples were taken out of the flask and added to the vessels in turn and the course of the reaction followed. By this means the combined effect of the products of the reaction and the destruction of trypsin is obtained. In order to distinguish the two effects another series of samples were taken at the same time as the above and immediately run into bottles standing in boiling water, which were at once corked. The next day this series was added to caseinogen in vessels as above and freshly made trypsin added, so that the trypsin content was that of the first series, supposing that no destruction had taken place during the digestion. This latter series gives the effect of the products of reaction, and if the rate of change is greater than in the former series, trypsin has disappeared during the digestion in the store flask. It would take too much space to give details of these experiments of which

¹ Journal of Physiology, XXVIII., p. 378.

² Archivio di Fisiologia, loc. cit.

several were performed. The results were not quite as regular as could be wished, but perhaps as much so as possible considering the number of sources of error. In one experiment there was no evidence of disappearance up to $7\frac{1}{2}$ hours, in another none up to $5\frac{1}{2}$ hours, a slight effect at 7 hours (this is the one of which curve figure 1 is the record). On the whole there is no evidence of disappearance up to 6 hours and few of my experiments were continued beyond that time. We are unable, then, to ascribe to this circumstance the rapid falling off of the velocity of reaction from that of a uni-molecular reaction.

When a digestion is carried on for two days there is evidence of trypsin destruction, viz., amount of change as 23 to 38, that is about 60% has disappeared. I do not lay much stress on this number, however, because the experiment was made with gelatin, and trypsin which has been warmed for some time, as I shall show later, has a peculiar effect in diminishing the conductivity of a gelatin solution in the early stages of the reaction.

From a practical point of view it is of importance to find out whether trypsin solutions lose activity rapidly at room temperature. I took a 2% solution of Grüber's trypsin, part of it was used at once and other fractions kept for 24 hours at 38° , 26° , 16° , 0° and frozen solid (-14°) respectively. Unfortunately I used gelatin to test their activities, unaware at that time of the above-mentioned effect of warmed trypsin on this body. The following are the values obtained:

Fresh solution	+ 552 gemmhos in 100'
Frozen at -14°	+ 500 "
Kept at 0°	+ 395 "
+ 16°	+ 184 "
+ 26°	- 103 "
+ 38°	- 90 "

These results serve to show the great instability of trypsin solutions even at 0° and, to some extent, when frozen solid. Tammann¹ states that emulsin in the dry state slowly loses activity and this I can confirm as regards trypsin. I am unable to give quantitative results, but a sample of Grüber's trypsin which I had kept in a stoppered bottle for some months was distinctly and unmistakably less active than when fresh.

I do not find the preparation of trypsin used by me to be as sensitive to heat as Vernon found his glycerin extracts of pancreas. In presence of 0.4% Na_2CO_3 he found 70% of the trypsin destroyed in one hour. In my experiments a 2% filtered solution of the "Rhenania" preparation was scarcely

¹ Loc. cit.

diminished in activity by one hour's exposure to a temperature of 39° . The difference may be accounted for, perhaps, by the fact that Vernon's preparation contained 0.4% of sodium carbonate; he also noticed that different preparations were markedly different as to their stability. I found that the addition of an equal volume of $\frac{n}{10}$ ammonia to my solution did not greatly increase the rate of destruction at 39° , the ratio of activity being as 20.2 to 21.65. But since the effect of alkalies is, no doubt, a question of the concentration of OH ions and ammonia is very slightly dissociated, viz., only to $\frac{1}{88}$ of that of sodium hydrate, the action of my $\frac{n}{10}$ ammonia would be scarcely comparable to that of 0.4% sodium carbonate. According to the measurements of Schields¹, $\frac{1}{20}$ molecular normal sodium carbonate is $\frac{1}{200}$ normal as regards OH ions, so that my solution would possess only about $\frac{1}{8}$ th the concentration of Vernon's as regards OH ions.

It is not easy to state what is the cause of this instability: we do not as yet know enough about the chemical structure of trypsin. If it has a proteid nature, it is possible that some sort of auto-digestion may take place, and the fact that destruction still proceeds at 0° to some extent supports this view, since, as will be seen later, trypsin is capable of slowly attacking caseinogen at that temperature. On the other hand the fact that the presence of proteid or its products of decomposition has so great a preservative effect suggests the formation of a compound with these bodies which is relatively stable.

The curious effect of warming trypsin as shown by its action on gelatin (of which curve A, figure 2, is an example), is, at first sight, very puzzling. This curve shows the fall of conductivity which is the first effect of the action of a 2% solution of Grüber's trypsin which has been kept at 26° for 22.5 hours. It was added to a 5% gelatin solution in the proportion of 1 c. c. to 10 c. c. of the gelatin. Curve B represents the effect of another part of the same trypsin solution kept at 16° , it will be seen that the change in the properties of the solution had not proceeded so far. It appears to me that this phenomenon may best be accounted for in the following way. The first effect of trypsin being the production of a compound with the substrate would probably be accompanied by a diminution of conductivity. Under the usual conditions, however, this intermediate compound would be split up again with great rapidity and would be masked, as far as its effect on the conductivity goes, by the rapid increase of conductivity accompanying the splitting up of the substrate. The first effect of heat on trypsin, I venture to

¹ Zeitsch. f. physik. Chemie. Bd. XII. S. 167, see also Kanitz. Zeitsch. f. physiol. g. Chemie. Bd. 37, p. 79.

suggest, is its conversion into a body analogous to the "toxoids" into which toxins can be converted by prolonged storage or warming. In the language of Ehrlich one would say that the toxophore or zymophore group was first destroyed, leaving the haptophore group, by which the enzyme attaches itself to the proteide molecule, unaffected¹. The formation of the compound of "zymoid" and substrate would still take place on adding this body to gelatin, but the decomposition of the substrate would proceed with greatly reduced velocity owing to the considerable weakening of the enzyme action of the trypsin. That the effect in question is not merely due to the diminu-

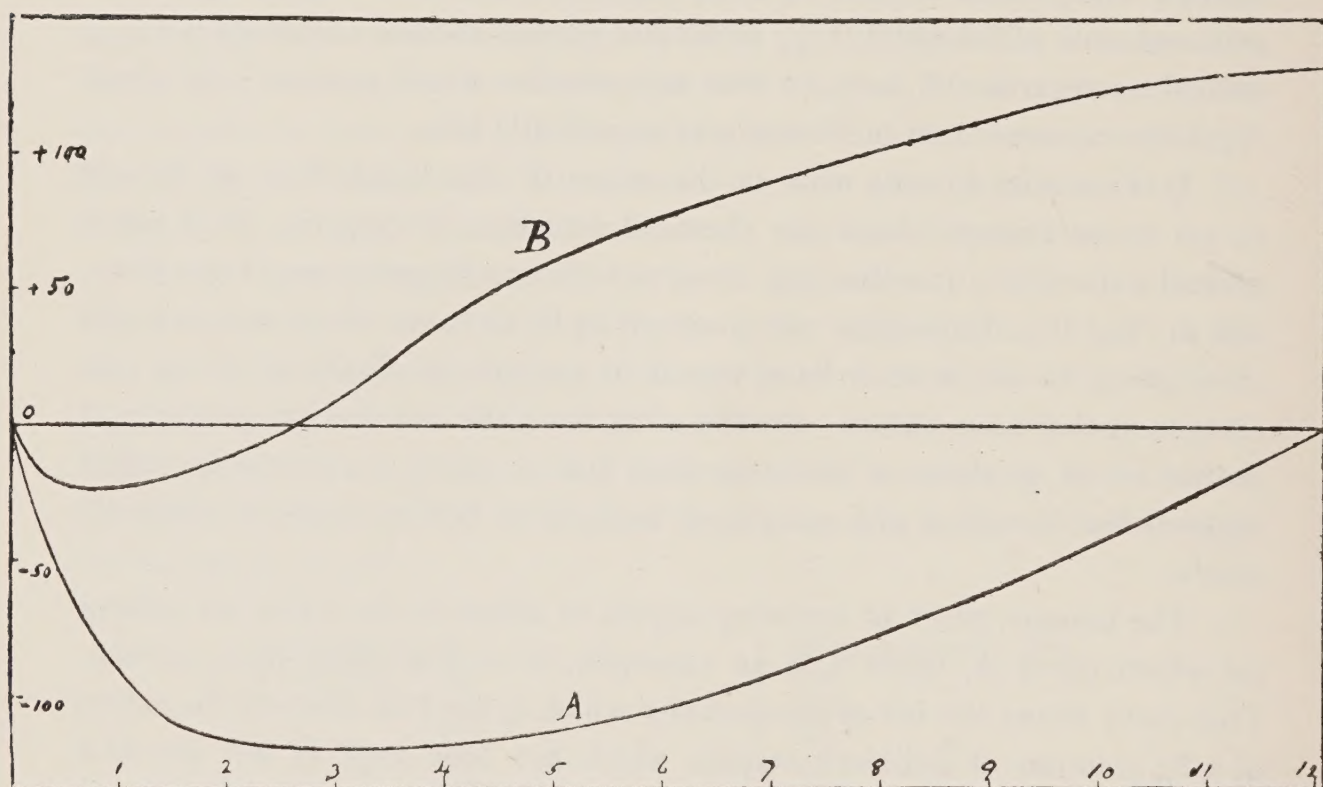


Fig. 2.

Ordinates—changes of conductivity in gemmhos.
Abscissae—time in hours.

tion in concentration of the trypsin is demonstrated by the fact that it is not shown by very dilute fresh solutions; these show only a very slow gradual increase of conductivity.

I am unable, as yet, to produce this phenomenon at will; it appears to be requisite that a certain relative concentration of the "zymoid" and the substrate should exist, and also that the substrate should not be too easily attacked. It is difficult to obtain with caseinogen, for example, though I

¹ If this view be correct, the injection of the "zymoid" into animals should be as effective in producing "antitrypsin" as the normal trypsin. This I hope to test by experiment.

have seen, in two cases in which I had a preparation showing a marked effect with gelatin, that there is an indication of an effect on caseinogen, shown by a dipping of the curve at the time when the gelatin curve was at its lowest point. I propose to study the action on such bodies as eggwhite, and also at a lower temperature of digestion, at which the tryptic action will be slowed, though, perhaps, the combining activity may be, relatively, less slowed.

I have no data to bring forward as to the velocity of the auto-destruction of trypsin. Tam mann¹ states that emulsin follows the logarithmic law, but, I think, his figures are not very convincing².

To return to our typical velocity curve, we have seen that, as regards the first six or seven hours, the disappearance of trypsin does not come into consideration. So that we now pass on to what is no doubt the chief factor in the divergence of the curve from a logarithmic one, viz., the influence of the products of the reaction.

4. The Effect of the Products of the Reaction.

That these products as a whole have a very considerable retarding effect can be shown in the way described above with respect to the destruction of trypsin, the second series, boiled samples to which fresh trypsin and caseinogen are added, being the object of study in the present case.

The following table gives values of change in the first hour, in gemmhos, under the influence of the total products (plus the unaltered substrate) taken from the experiment of curve 1 at the times given.

Sample taken at	Change in 1 hour
3'	1000
13'	1245
36'	790
71'	670
151'	550
222'	420
324'	435
448'	420

It is of interest to compare these with the corresponding series taken by adding the samples at once (without heating) to caseinogen.

Sample taken at	Change in 1 hour
7'	1360
15'	1370
39'	1110
74'	840
154'	715
225'	500
317'	490
441'	500

¹ Loc. cit.

² Vide Senter. Zeitschr. f. physik. Chemie. XLIV, p. 295.

Of course these numbers are not exactly comparable one with another, because more unaltered caseinogen is added with the earlier ones than the later, and this would affect the rate of change in the same direction as that of the products of reaction. Supposing that in the first sample none of the caseinogen was yet attacked, then the final mixture would contain 5%, and supposing that in the last sample there was none left unattacked, the concentration would then be 2.5%, and as far as this factor is concerned the rate of change would be as 380 to 200 approximately, that is the final value might be $1370 \times \frac{200}{380} = 721$, that is 1.5 times that of the actual value found, and it is also to be remembered that there was, at the end of this particular experiment, a considerable amount of caseinogen left unattacked. To obtain strictly correct numbers, however, it would be necessary to determine the unaltered caseinogen by some chemical method at intervals and add to the tests a corresponding amount to make all equal in caseinogen content.

It will be noticed that the three last samples are about equal in their retarding power, corresponding to the flatness of the typical velocity curve at this stage. The reason why the second sample has a slightly less retarding power than the first I am unable to state; possibly it is connected with the production of "metacasein"¹, which is coagulated by heat and it is interesting to note that the effect in question is more marked in the heated samples. The retarding effect of the products can be shown also without the concurrent alteration of concentration of the substrate by taking a solution of the products of a prolonged digestion, so that none of the original substrate is present, thus:

A contains Caseinogen 5% 4 c. c. + 5% solution of products 5 c. c. + $\frac{n}{2}$ NH₃ 1 c. c. + 2% trypsin 1 c. c.

B contains a similar mixture but with 5 c. c. of water instead of the digestion products.

After 1½ hours the amount of change was:

A	540	gemmhos
B	1660	„

Before proceeding to further analysis of this phenomenon, it will be well to discuss briefly the nature and cause of the increase of electrical conductivity in tryptic digestion. It is easy to show that it is not merely a matter of diminution of internal friction. The curves of figure 3 are concurrent measurements of the internal friction, by Ostwald's viscosimeter (curve A),

¹ William Roberts. Proc. Royal Soc., London, 1881.

and electrical conductivity (curve B), in the case of a caseinogen-trypsin experiment. The ordinates of the viscosity curve (A) are inversely proportional to the times of flow through the capillary tube of the instrument. What we see is, that the internal friction diminishes with great rapidity at the commencement of the reaction, but becomes constant while the conductivity is still increasing fairly steeply. The curve of conductivity at the beginning may, perhaps, be influenced by the rapid decrease of internal friction. This rapid diminution in viscosity at the commencement was also noticed by Spriggs¹ in the case of pepsin action, his curves, however, do not become horizontal at so early a stage in the reaction as in the case before us.

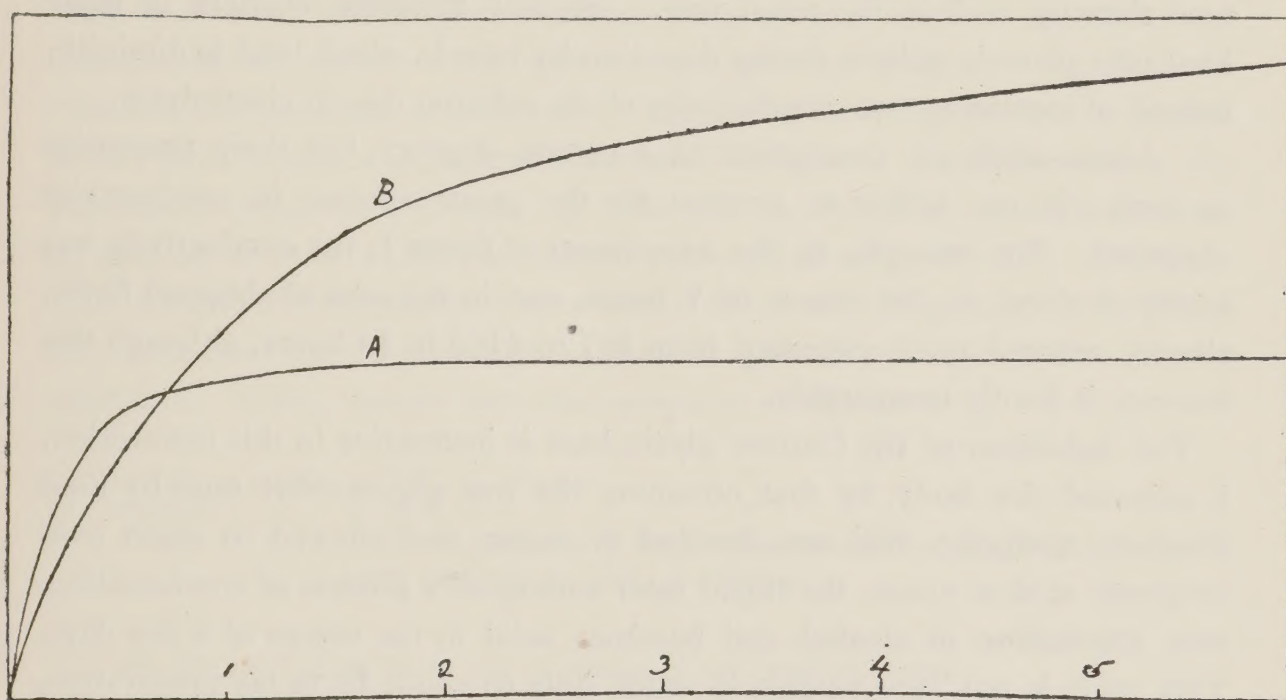


Fig. 3.

The following result also shows that, in the case of caseinogen, changes in the physical state of the solution do not appreciably affect its relation to electrolytes. A solution of caseinogen was taken and trypsin added, part of it was heated to 100° at once, and the other part was kept at 39° for some days. An equal amount of $\frac{n}{10}$ KCl was added to both and the increase of conductivity was as follows :

In the undigested caseinogen	1380 gemmhos
„ „ digested „	1400 „

¹ Zeitschr. f. physiolog. Chemie. Bd. 35. S. 465.

Gelatin behaves differently ; it has been mentioned already that the presence of gelatin in a solution does not, to any degree worth consideration, affect its electrical conductivity. The products of its digestion, on the contrary, diminish the conductivity of a solution containing electrolytes. A similar experiment to that above was performed on gelatin with the following result :

Undigested gelatin	6760	gemmhos
Digested	„	1266 „

In the undigested mixture, in fact, the conductivity was, practically, the sum of that of the gelatin mixture and that of the KCl ; in the digested, on the contrary, it was far less, owing to the influence of the non-electrolytes now showing itself in the usual way. So that, therefore, changes of some kind take place in gelatin during digestion by trypsin which tend to diminish, instead of increasing, any conductivity of the solution due to electrolytes.

Amino-acids are conductors to a certain degree¹, but their properties as such will not suffice to account for the great increase in conductivity observed. For example, in the experiment of figure 1, the conductivity was nearly doubled in the course of 7 hours, and in the case of chopped fibrin, already referred to, it increased from 287 to 4145 in 18 hours, although this instance is hardly comparable.

The behaviour of the Curtius' glycin-base is instructive in this connection. I prepared this body, by first obtaining the free glycin-ethyl ester by Emil Fischer's method ; this was distilled in vacuo and allowed to stand over sulphuric acid in vacuo, the liquid ester undergoes a process of condensation, with elimination of alcohol, and becomes solid in the course of a few days. This solid is not very soluble in water, only to about 1% in my preparation. I took 80 c. c. of this 1% solution, which would contain no electrolyte, beyond what was present in the water ; the water was not a very good sample, indeed, having a conductivity of 31 gemmhos. To this solution I added 5 c. c. of $\frac{n}{2}$ ammonia and 10 c. c. of a 2% filtered solution of trypsin "Rhenania." The conductivity was now 1575 gemmhos, and gradually increased, following a curve like fig. 1, attaining a value of 2500 gemmhos in two days. At this time there was still a marked biuret reaction, so that a considerable part of the base was yet unattacked. I refer to this experiment here in order to show that the production of mon-amino-acids may partly account for the increased conductivity in the case of caseinogen. It appears, however, that in the case of gelatin, amino-acids are not produced in any appreciable quantity, at all events not within the first two hours of the action

¹ See Kohlrausch and Holborn. Loc. cit. p. 176.

of trypsin. We must then look for other causes. One of these, viz., the effect of change of physical state, has been already mentioned. The point next suggesting itself is that concerning the inorganic constituents of these proteid and related bodies. The balance of evidence seems to be decidedly in favour of the view that these constituents are, if not actually in chemical combination with the proteid molecule, in such a close state of association as to be incapable of ionization. It has not been found possible hitherto to prepare an unaltered proteid free from ash. No doubt a considerable amount is usually present as impurity which can be separated by prolonged dialysis. If, then, inorganic constituents exist as part of the proteid molecule, they will, in all probability, be set free by the action of trypsin, and, being ionized will then be able to manifest their characters as good conductors. The following experiment will throw a little light on this obscure question. A 5 per cent solution of commercial gelatin had a conductivity of 765 gemmhos; a portion of this same gelatin was soaked in repeated changes of distilled water, in the ice-chest in presence of thymol, for some five or six weeks; it was then dried by soaking in alcohol and exposure to a current of warm dry air. A five per cent solution of this gelatin had a conductivity of 160 gemmhos. That of the water used was 68 gemmhos, so that the gelatin itself possessed only 92 gemmhos. Three vessels were then prepared as follows:

- A. Ordinary gelatin + NH_3 + trypsin
- B. Washed " + NH_3 + "
- C. " " to which sufficient $\frac{n}{10}$ KCl had been added to bring its conductivity up to that of A + NH_3 + trypsin.

At the end of an hour the increase of conductivity was:

- A. 1300 gemmhos
- B. 1310 "
- C. 1200 "

It seems to me that the only conclusion to be drawn from this result is that the only electrolytes taking part in the increase of conductivity were those previously present as constituents of the gelatin. Probably the slightly lower value of C was due to the retarding action of the free electrolytes present.

Another experiment of interest here is that with fibrin already referred to. This fibrin had been thoroughly washed. In the process of digestion the conductivity of the solution in which it was present rose by 3860 gemmhos. At the end the whole was incinerated in a platinum capsule, taking care that the temperature did not rise above low red heat. The

weight of the ash was 0.0111 grm. Now, as a rough approximation, if we take this ash to consist of calcium chloride it would account for an increase of conductivity of 3780 gemmhos. So that the inorganic constituents previously combined with the fibrin are capable of accounting for the increase of conductivity, so far as this very inadequate experiment goes. It would be of considerable interest to know the behaviour of Harnack's "ash free albumin" under the action of trypsin; this I hope to study.

It is also to be borne in mind that certain proteids, as caseinogen, split off their organic phosphorus as inorganic phosphates. I have made some determinations of this change, using Neumann's¹ method. Unfortunately, by an oversight, the amounts of solution taken were too small, so that only in the later stages were the results of any value. At the end of the curve of figure 1 there were present 9.2 milligrams of P_2O_5 per 100 c. c. of the solution. These determinations must be repeated with larger quantities of caseinogen solution.

Now it is known that, in certain cases, electrolytes have a marked depressant effect on the action of enzymes, for example 1% $CaCl_2$ reduces the action of amylase starch to one-half². As regards neutral salts, the effect, however, is not the same in the case of different enzymes. It may, nevertheless, be worth while to test whether the production of electrolytes during tryptic digestion is capable of accounting in any way for the action of the products of reaction. I took solution of caseinogen, determined its conductivity, added KCl until the conductivity was doubled (to correspond with the result of a seven hours digestion); it was necessary to add 3 c. c. of $\frac{n}{10}$ KCl to 9 c. c. of the caseinogen to do this. Another sample was taken to which potassium phosphate was added in the quantity shown by the above determination to have been produced at seven hours, the acid phosphate was used, and, before addition, it was made, as regards its reaction, just acid to phenolphthalein but alkaline to litmus. As control a similar caseinogen solution was brought to the same volume as the previous two by addition of water. Ammonia and trypsin were added to all and the amounts of change, at the end of two hours, were as follows:

Water only	1835 gemmhos
Potassium chloride 0.017%	1790 ..
.. phosphate ..	1805 ..

The effect is very slight indeed, 2.4% reduction, almost within the limit

¹ Zeitschr. f. physiol. Chemie. Bd. 37, p. 129.

² Effront. Les Enzymes. p. 138.

of experimental errors, and by no means capable of explaining the much greater effect of the total products of reaction.

Although neutral salts, in physiological concentrations, have but little effect on trypsin, it is well known that it is very sensitive to the OH ions of alkaline solutions. An example of ammonia, in decimolecular solution, may serve to illustrate this fact :

Solution of caseinogen 2.5% containing of $\frac{n}{10}$ ammonia in 11 c. c. :	Amount of change in 1 hour.
5 c. c.	1770 gemmhos
4 „	1565 „
3 „	1365 „
2 „	1120 „
1 „	680 „
0 „	319 „

This being so, is there any change of alkalinity during digestion to be reckoned with? By the ordinary methods of titration I have been unable to detect change in 24 hours, but I am at present engaged in investigating the matter by the more delicate method of measuring the potential differences with hydrogen electrodes.

When we pass to the effect of the organic non-electrolytes, such as albumoses and semi-conductors, like the amino-acids, we are met by considerable difficulties. It is very difficult to obtain samples of these various products in an even approximate state of purity ; this applies not only to the various albumoses and peptones but even to the amino-acids. As regards these latter bodies, no doubt the most satisfactory method of obtaining them free from electrolytes would be by the Emil Fischer method of distilling in vacuo the ethyl-esters and then hydrolyzing by prolonged boiling with water of lowest conductivity in Jena glass vessels. I have not yet had time to do this, and have at present used only the preparations of Merck and Grüber and a sample of glycine prepared by myself by boiling hippuric acid with sulphuric acid, the sulphuric acid being got rid of by barium carbonate, the solution evaporated, recrystallized and finally washed with alcohol and ether ; this preparation contained a trace of barium, as shown by the faint cloud produced in its solution on the addition of sulphuric acid.

The first question that suggests itself is whether there is any specific action of the products or any particular family of them. I give below the results of an experiment with various non-electrolytes and glycine (the hippuric preparation described above) all added in the proportion of 3 c. c. of a decimolecular solution of each to 8 c. c. of a 4% caseinogen solution with trypsin and 1 c. c. $\frac{n}{10}$ NH_3 .

SUBSTANCE ADDED.							Initial Con- ductivity.	Change in 20 hours.
								gemmhos.
Water	..	..	..	..	..	..	4630	1700
Ethyl alcohol	..	..	..	..	..	..	4650	1590
Glycerin	..	..	..	..	..	..	5000	1660
Cane sugar	..	..	..	..	..	..	4908	1550
Glucose	..	..	..	..	..	..	4900	1570
Urea..	..	..	..	..	..	..	5000	1640
Asparagin	..	..	..	..	..	..	5133	1050
Glycin	..	..	..	..	..	..	5600	1540
Gum. arabic 27.56%	..	..	..	..	..	..	5000	1570

The strength of the gum-arabic solution was isotonic with the others according to Pfeffer's determination (Osmotische Untersuchungen, p. 75).

There is, it will be noticed, very little effect with any of these bodies, with the exception of asparagin, and what effect there is, a slight depressant one, is practically the same in all, that is, proportional to the number of the molecules present, or to the osmotic pressure. Perhaps more marked differences may be found with more concentrated solutions, but this experiment, so far as it goes, does not indicate any particularly marked effect of amino-acids.

The next experiment was made with equal content by weight of various bodies of direct interest; not knowing the molecular weight or osmotic pressure of all these bodies, each was added in 5% solution in the proportion of 4 c. c. to 6 c. c. of the caseinogen-trypsin mixture.

SOLUTION ADDED.							Initial Con- ductivity.	Change in 1 hour.
Glycin (Merck)	..	..	..	..	..	..	5700	380
Leucin "	..	..	..	..	..	..	4310	500
Amphopepton (Grübler)	..	..	..	..	..	..	6860	870
Deuteroalbumose (Grübler)	..	..	..	..	..	..	11160	1120
Products of a tryptic digestion of caseinogen still giving biuret	..	..	..	..	..	..	7210	690
A-biuret products of a similar digestion	..	..	..	..	..	..	8800	575
Albumoses from a tryptic digestion, prepared by ammonium sulphate	..	..	..	..	..	..	6070	680
Cane sugar (control)	..	..	..	..	..	..	2905	1295

It is evident from the initial conductivities that the preparations of albumoses and pepton can hardly be looked upon as pure, though it is evident that as digestion proceeds the products become more and more poisonous. Deutero-albumose is scarcely comparable with the rest, moreover, since it is attacked fairly rapidly by trypsin and would therefore increase the concentration of the substrate.

It does appear, however, that, supposing a given amount of caseinogen is split up into an equal amount of products, as of course happens, these products would exert a greater and greater retarding effect as digestion proceeds. In other words, given an equal mass of peptones and albumoses on the one hand and amino-acids on the other, the effect of the latter is far greater. As to how far this is due to increased osmotic pressure or to any specific action is a matter for further investigation.

Victor Henri¹ finds that, in the case of invertase, the same quantity of invert-sugar exerts a greater action on small concentrations of cane-sugar than on greater ones. This does not seem to be the case with trypsin, however, as the following experiment shows:

Six vessels were taken and solutions of the following composition added to each:

A.	5%	Caseinogen	4 c. c.	+	5%	Products	5 c. c.	+	$\frac{n}{2}$ NH ₃	1 c. c.	+	trypsin.
B.	"	"	2 "	"	"	"	5 "	"	"	"	"	"
C.	"	"	1 "	"	"	"	5 "	"	"	"	"	"
D.	"	"	4 "	"	"	"	0 "	"	"	"	"	"
E.	"	"	2 "	"	"	"	0 "	"	"	"	"	"
F.	"	"	1 "	"	"	"	0 "	"	"	"	"	"

All made up to same volume with water. Amounts of change in $2\frac{1}{2}$ hours were:

A.	720	gemmhos.
B.	550	"
C.	310	"
D.	1985	"
E.	1370	"
F.	773	"

In the controls D, E and F the ratio of the amounts of change was as 2.57 : 1.37 : 1; in the samples with products the corresponding ratios were 2.33 : 1.75 : 1. Put in another way, the ratio of the difference between A and D to the difference between B and E and to that between C and F were as 5.68 : 3.68 : 1.

The experiments with the Curtius' biuret-base have not, as yet, turned out so satisfactory as was hoped. Trypsin, although it does undoubtedly act upon it, is not very active in this respect, so that, in the prolonged experiments necessary, there is much destruction of the enzyme. How far trypsin is protected by the base, or glycine itself, I am unable to state. In the investigation of the rate of change, I made use of, in addition to the conductivity method, that of spectro-photometric observations of the biuret reaction according to the method of Klug². The instrument used was that

¹ Archivio di Fisiologia, loc. cit. p. 314.

² Pflüger's Archiv. IX., p. 43.

of d'Arsonval as supplied by Mr. Pellin, of Paris. The region of the spectrum taken was that between the wave-lengths 540 and 531 $\mu\mu$. The values of the expression $\log. \frac{I_0}{I}$, where I_0 is the entering light and I the issuing light, as read off on the scale of instrument, were used as the basis for the construction of curves. When a 1% solution of the base is taken and trypsin added in the proportion of 20 c. c. of a 2% solution to 100 c. c. of the substrate, it is found that there is a regular increase of the conductivity forming a curve like that of caseinogen or gelatin. The biuret reaction, however, contrary to expectation, for the first four or five hours increases and then slowly decreases. This is not due to the trypsin itself, for, in the case of the "Rhenania" preparation used, it did not itself give a biuret reaction in the concentration in which it was present in the solution in question. In 2% solution it gave a very slight biuret colour that was increased by keeping the solution at 38°. I suggest, with considerable hesitation, that this first increase of biuret reaction in the case of trypsin acting on Curtius' base may possibly be due to the preliminary combination of the enzyme and substrate; although, if this be so, it is an interesting fact that the biuret reaction of the base should be increased in the process. There is, moreover, no evidence of a synthetic process in the electrical conductivity. I may say that I am not altogether satisfied with the technique of the biuret reaction. It takes a certain time to develop, as Vernon¹ points out, and seems to go on slowly increasing for some time. There is also a certain cloudiness produced in the solution, which has, therefore, to be carefully filtered, so that there are uncontrollable sources of error present. It is to be hoped that these sources of error may be eliminated, since the method is very useful, and may be applied to the other colour reactions of proteids, etc., under tryptic digestion.

A final point to be referred to as to the effect of the products of reaction is in connection with their mode of action. There is some evidence that they form a combination with the enzyme in the fact that they so effectively protect the latter from destruction. An experiment may be mentioned here in which trypsin solution alone, the same solution with the addition of caseinogen, and again with the additions of products of reaction, were heated for 10 minutes to 64°. These were tested subsequently by allowing them to act on caseinogen at 38°. The changes in four hours were as follows:

Trypsin heated alone		650 gemmhos.
" " with caseinogen		675 "
" " " products of reaction	1105	"

¹ Journal of Physiology, XXX., p. 331.

In this case the protective effect of the products was greater than that of caseinogen. It would be interesting to raise the temperature still higher so as to destroy the free trypsin, since it is stated by O'Sullivan and Thompson¹ that invertase in the presence of cane-sugar will withstand a temperature fully 25% higher than in its absence.

As regards the specific nature of the retarding action of the products on the rate of change, it is of interest to compare the effect of the products of gelatin digestion on caseinogen and vice versa. An experiment I made showed that the effect of gelatin products on the digestion of both itself and caseinogen was considerably more marked than that of the products of caseinogen on either itself or gelatin; but the question requires further investigation, since it is difficult to know how far the products in two cases can be considered to be those of corresponding stages in the two reactions, and it would perhaps be better to take bodies more nearly related than gelatin and caseinogen, say albumin or fibrin and caseinogen.

In connection with the apparent non-retarding effect of certain colloids on the velocity of reaction, already referred to above, an experiment with starch is worth quoting. A 3% starch paste was taken, a small quantity of Taka-diastrase added, a portion of the mixture immediately heated to 100° and the rest allowed to remain for 24 hours at 38° and then heated to 100°. These were added in quantities of 5 c. c. to 5 c. c. of 5% caseinogen, a third (control) being with 5 c. c. of water in which 3% starch had soaked in the cold. Trypsin and ammonia were added and the changes in 1½ hour compared :

Caseinogen and starch-paste	870 gemmhos
" " " acted on by diastase	660 "
" " water as above	1330 "

The raw starch-paste had therefore considerably less retarding action than that converted to sugar by diastase, but itself had a very marked effect.

5. Concentration of Enzyme.

The law of mass-action states that

$$-\frac{dC_{\text{subs.}}}{dt} = KC_{\text{subs.}} \cdot C_{\text{enzyme.}}$$

Now we have seen that, in moderate concentrations, the rate of change is proportional to the concentration of the substrate and we will proceed to discuss the effect of changes of concentration of the enzyme.

¹ Trans. Chem. Soc., Vol. 57, p. 919, 1890.

Various statements have been made as to this relation in the case of enzymes, and this conflict is, no doubt, due to the fact that the relation between rate of change and concentration of enzyme is not the same at all stages of the reaction¹. In fact, if we consider for a moment the peculiarity of catalytic action, viz. that considerably different proportions of catalyzer are able to effect the same final change, if only the smaller quantities are allowed time enough, and the catalyzer remains in itself constant, we see that the above-mentioned fact is a necessary consequence.

In the experimental study of this aspect of our problem, I have taken, as criterion of relative activity of different proportions of trypsin, the reciprocal of the time in minutes required to effect the same absolute amount of change in the electrical conductivity of the substrate solution. By this means we may assume that we are dealing with corresponding stages of the reactions, so that there will be the same relative concentration of the products of the reaction, etc., and the more complex function involved in taking the amount of change in equal times is eliminated².

As a first example of an actual experiment we will take the following. A series of six vessels each supplied with 5 c. c. of 5% caseinogen and 1 c. c. $\frac{n}{2}$ NH_3 were taken and varying amounts of 2% trypsin added to the series viz. 4, 2.5, 2, 1, and 0.5 c. c. the appropriate amount of the same trypsin, previously heated to 100° , having been previously added to each so that the final volume was the same (11 c. c.) in all. The times taken in each for the changes of conductivity given at the head of the columns were as follows :

Relative trypsin content.	Time in minutes required for a change in gemmhos of						
	0—800	0—1300	0—1800	0—2200	800—1300	1300—1800	1800—2200
4	4.5	13	54	266	8.5	21	212
2.5	7.5	19	58	245	11.5	39	187
2	10	24	79	274	14	55	195
1	19	45	126	480	26	81	354
0.5	37	89	233	720	52	144	487

This table teaches us several things of interest :

1. The change in the initial stage is in linear proportion to the amount of enzyme.

¹ See the valuable "Studies on Enzyme Action" by E. Frankland Armstrong. Proc. Royal Soc., Vol. 73, No. 496, 1904. It is my misfortune not to have had these papers before me during the whole of my work.

² See Bredig. *Ergebn. d. Physiologie*. Erster Jahrgang. I Abt. 1902, S. 159.

2. This relationship is more and more departed from as the reaction proceeds.

3. Between the third and fourth stages of the table (1800—2200 gemmhos) the rates of changes are practically identical for the first three strengths of enzyme. For the lower concentrations of trypsin the results are not strictly comparable because by this stage there had probably already been an appreciable destruction of the enzyme.

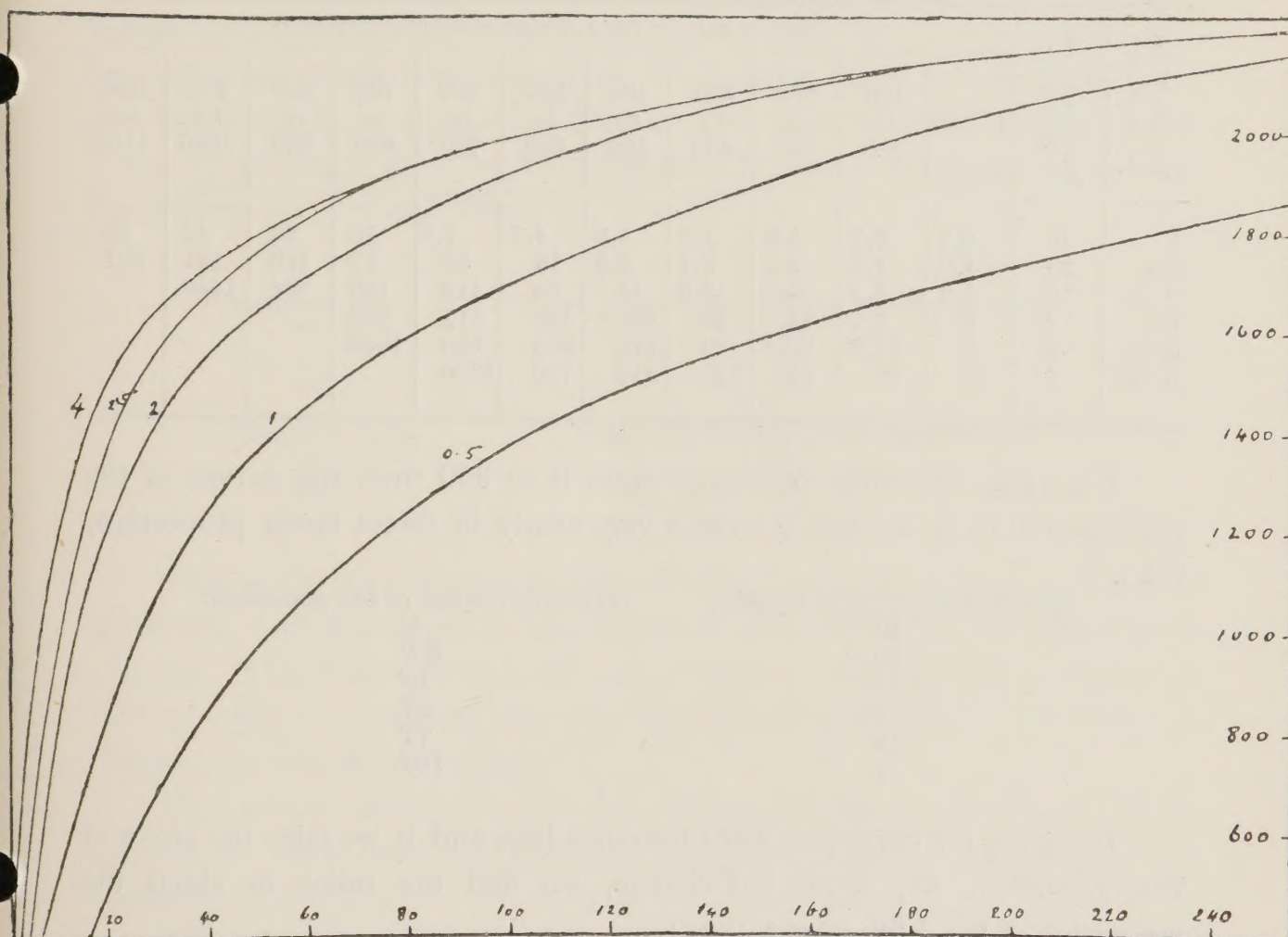


Fig. 4.

The curves of fig. 4 represent a portion of this experiment. The divisions on the axis of abscissae denote time in minutes, and those on the axis of ordinates conductivity changes in gemmhos reckoning from the beginning of the curves which, to save space, is omitted. It is interesting to note how the four upper curves join together and take their further course in common.

The probable meaning of the facts here brought out will best be discussed in speaking of the mode of action of trypsin.

A similar experiment with gelatin is given in the table below.

Smaller proportions of trypsin were taken, and the facts shown in the previous experiment do not come out so clearly. The early readings in the case of gelatin are not usually so regular as in the case of caseinogen. The amounts of trypsin added in the six cases were 1, 0.5, 0.25, 0.1, 0.05, 0.025 c. c. respectively, to 10 c. c. 5% gelatin. It was, however, a more active preparation than the previous one.

Trypsin 1% in c. c. added.	Relative amounts of trypsin.	Time in minutes for a change of conductivity from										
		0—100	100 to 200	200 to 300	300 to 400	400 to 500	500 to 600	600 to 700	700 to 800	800 to 900	900 to 1000	1000 to 1100
1	40	0.5	0.7	1.3	1.4	1.6	4.6	9.8	16	26	46	69
0.5	20	1.0	1.5	2.3	3.7	8.5	13	33	52	110	191	654
0.25	10	1.0	1.4	3.5	8.5	14	38	112	180	294	1440	
0.1	4	6	6.7	14	36	105	192	511	924			
0.05	2	9	11.5	30.5	61	173	365	690	1560			
0.025	1	12	29	54	155	312	720	1500				

If we take the times of change from 0 to 350 from the curves of the experimental data, we find the effect very nearly in direct linear proportion, viz. :

Relative amounts of trypsin.	Times of change of 350 gemmhos.
40	3'
20	6.5'
10	10'
4	40'
2	75'
1	150'

Later on, the correspondence becomes less, and if we take the times of 725 gemmhos, *i.e.*, about half-change, we find the ratios in about the proportion of the 1.5th power, thus :

Trypsin content.	Times of change of 725 gemmhos.
40	22.5'
20	75' = 22.5 × 2 ^{1.42}
10	218' = 22.5 × 4 ^{1.64}
4	1051' = 22.5 × 10 ^{1.67}
2	1634 = 22.5 × 20 ^{1.43}
1	> 2814

A logarithmic stage therefore follows the linear one.

In another experiment with quantities of trypsin as 10 to 1 acting on caseinogen the velocity constants calculated for the first stage of the action were as 0.01657 to 0.0016.

6. The temperature-coefficient of the Reaction.

Since very few measurements of the velocity-quotient in the case of enzymes have been made, it may be worth while to put on record the results of an experiment with trypsin. Samples of the same mixture of caseinogen, ammonia and trypsin were allowed to digest at the temperatures: $20^{\circ}.7$, $30^{\circ}.7$ and $38^{\circ}.7$, in three different thermostats. The times of equal change were determined by the conductivity method as usual. In order to compare the results it is necessary to correct the conductivity readings by obtaining the temperature-coefficient of the solution. This was found to be 66 gemmhos for 10° ; the initial conductivity at $30^{\circ}.7$ being 3550 gemmhos, the percentage increase per degree is 1.36, a number approximating closely to the value, 1.8 per cent., given by Arrhenius for OH ions¹. The results, corrected for temperature, are as follows:

Temperatures.	Times required for change of 930 gemmhos.
$20^{\circ}.7$	360'
$30^{\circ}.7$	68'
$38^{\circ}.7$	26'
Temperatures.	Times for change of 1600 gemmhos.
$30^{\circ}.7$	158'
$38^{\circ}.7$	60'

That is it takes 5.3 times as long to effect an equal change at $20^{\circ}.7$ as at $30^{\circ}.7$; and 2.6 times as long at $30^{\circ}.7$ as at $38^{\circ}.7$ or 3.3 times for 10 degrees. This latter value is the same whether calculated for a change of 930 or 1600 gemmhos so that the temperature-coefficient is the same at different periods of the reaction.

The smaller coefficient for the higher temperature ($30^{\circ}.7$ to $38^{\circ}.7$) appears to show that the enzyme is showing signs of approaching the optimum.

Tammann² found for emulsin between 60° and 70° a value for 10° of 7.14; and Senter³ for the hydrogen-peroxide splitting enzyme of blood between 0° and 10° the value of 1.5. In the table of Van't Hoff⁴ the quotients for various chemical reactions vary from 1.2 to 3.68.

I may again refer to the experiment in which it was found that trypsin was active on caseinogen at 0° . The vessel was kept in melting ice and in 5400 minutes there was a change of 1043 gemmhos at 0° . If we assume the

¹ Lehrbuch d. Elektrochemie (Trans. by Euler) 1901, p. 136.

² Zeitschr. f. physik. Chemie. Bd. 18, S. 436.

³ Zeitschr. f. physik. Chemie. Bd. 44, S. 292.

⁴ Vorlesungen. I. S. 225.

validity of the above temperature-coefficient of conductivity, viz., 1.86%, this will mean a change of 1325 gemmhos; and comparing with the time taken at $30^{\circ}.7$ for a change of 1600, viz., 158', as a very rough approximation, a velocity-quotient of 12 for 10° between 0° and 30° is obtained. It is evident that the activity is greatly reduced at 0° .

7. Anti-trypsin.

It is well-known that fresh serum and egg-white show considerable resistance to the action of trypsin, and, although this is attributed by some to mere difficulty of attack by the enzyme of the proteids present in these fluids, it is the more general opinion that there are specific anti-bodies concerned. My experiments support this latter view.

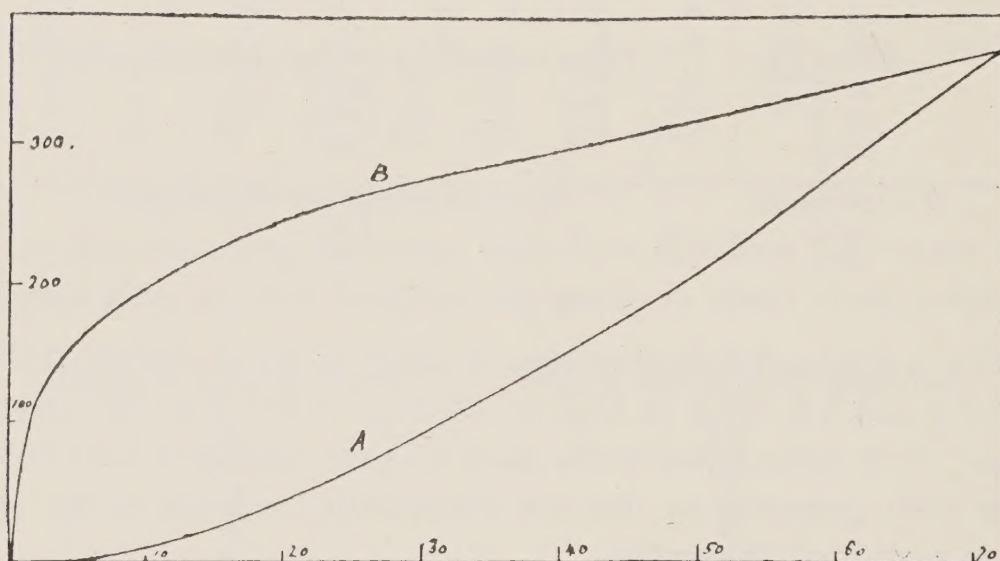


Fig. 5.

A 10% mixture of egg-white and distilled water is taken, to 10 c. c. there is added 1 c. c. of a 1% trypsin solution, another 10 c. c. is heated to 100° before adding the trypsin. As Hardy has shown, the solution becomes milky but does not coagulate. The curve A of fig. 5 shows the course of the conductivity change in the fresh solution and B that in the heated one.

It will be observed that while B shows the usual time curve, though slow, the times marked being hours, curve A does not rise at all for some four hours and takes a course convex to the axis of abscissae. Now it appears to me that this peculiar curve can only be explained by supposing the presence of an anti-trypsin which gradually disappears, as Dastre¹ has

¹ C. R. Soc. de Biologie, 1903, p. 634.

shown happens in the case of maceration of intestinal worms, and I have been able to confirm. A similar curve is obtained by the use of serum instead of egg-white. When caseinogen is added to a trypsin solution containing sufficient serum to completely prevent the attack of it by the trypsin for at least two days, it is found that the caseinogen is slowly attacked and the curve, in one case, became a straight line inclined at an angle upwards. The probable explanation of this circumstance of a certain degree of digestion of caseinogen, going on when serum itself is completely unaffected, is that the anti-body affects only the trypsin and not the weak proteoclastic¹ enzyme associated with it. This will be explained more fully when treating of natural juice. These facts, of course, do not decide the question of an anti-enterokinase in serum, they only show that an anti-trypsin is present, since the commercial preparations of trypsin contain no enterokinase.

8. Antiseptics.

I have not made any systematic study of the action of these on the rate of change. But for purposes of control I have tested the action of toluol and chloroform. Under the conditions in which I worked, there was no obvious effect of chloroform, and toluol had a slightly accelerating effect. This is of importance in prolonged experiments which are sometimes necessary. Compare the results of Kaufmann².

II. Equilibrium and Reversibility.

The impression given by consideration of the course of such a curve as that of fig. 1 is that it is distinctly tending towards a position of equilibrium, gradually becoming more horizontal. That this is not due to a disappearance of the trypsin can be shown by adding more substrate when we see the curve begin to rise again. The trypsin having become inactivated by combination with the products of reaction can probably, by mass-action, be dissociated from this combination, and a part of it combines with the substrate preparatory to the decomposition of this latter.

There is a certain amount of evidence that proteoclastic enzymes are capable of synthetic action. I may mention the "plastein" of Danilevsky

¹ I use the name suggested by Armstrong Chem. Soc. Trans., 1890, Vol. 57, p. 528. "Proteolytic" in analogy with "hydrolytic," ought to refer to splitting by means of proteid, not splitting of proteid.

² Zeitschr. f. physiolog. Chemie. Bd. 39, S. 452.

formed by the action of pepsin, trypsin and papayotin on concentrated solutions of albumoses¹. I have noticed also that the effect of intestinal extracts containing erepsin on 3% Witte peptone is to produce, in the course of two days, a considerable precipitate.

To test whether the conductivity method would give any indication of such a synthetic process I exposed a 40% solution of the products of reaction of caseinogen to the action of trypsin. There was, in fact, a slow diminution of conductivity lasting four days, which then returned in the course of a day or two to the original value. The amount of this diminution was not great, viz., 8200 gemmhos in 30000 gemmhos, but I think it is not to be expected that it should be so, since it is hardly likely that electrolytes would be taken up in the product formed and the change would be manifested only in an increase of viscosity, which, as shown above, has not a great effect on the conductivity, especially if due to increase of colloids.

Whatever view we may take of the apparent equilibrium referred to, viz., whether due to a reversible inactivation of the enzyme by the products of its activity or otherwise, it is certain that we can alter its position by similar changes as, according to mass-action, are active in ordinary reversible reactions. Such changes are :

1. Adding more substrate.
2. Adding more trypsin. It is not to be expected, however, that any considerable effect will be thus produced, since there is very little unaltered substrate left when "equilibrium" is attained.
3. Dilution or concentration. I have found dilution to cause a further action, but have not tested concentration.
4. Removing products of reaction. By dialyzing an advanced tryptic digestion, concentrating to reduce to original volume and adding trypsin to make content of enzyme equal to that of the original fluid, it is found that a further action can be obtained.
5. Raising the temperature also appears to cause further change, but it is difficult in using the conductivity method to eliminate the effect of temperature on the actual conductivity itself.

The fact that adding enzyme changes the position of "equilibrium" shows that we have to deal with relations not merely between the substrate and its products of decomposition, as in the case of acetic acid, alcohol, water, and ethyl acetate, but more complex ones in which the enzyme also is a factor.

¹ See Herzog. Zeitschr. f. physiolog. Chemie. Bd. 39, p. 305.

III. The Question of Multiple Products.

My work on this part of the subject is as yet only in its commencement. But some results obtained are perhaps of interest.

In taking the electrical conductivity as an index of the changes going on, we must bear in mind that the splitting off of electrolytes may be only one of the various concurrent reactions produced under the agency of trypsin, so that others proceed with a different time law. As stated in the introduction, the investigation of the reaction velocity by other methods will throw light on this question.

The internal friction.—Figure 3 shows that this property does not change concurrently with the conductivity and has been already discussed.

The refractive index.—Measurements were made by Pulfrich's refractometer and slight diminution observed, but the effects were too small to admit of accurate measurement.

Rotation of the plane of polarized light.—Gelatin and caseinogen solutions are laevo-rotatory and under the influence of trypsin there is a regular diminution of the rotation in the form of a curve similar to that of the increase of conductivity. These experiments have been done at room temperature, but I am now arranging a warm-water circulation to the polarimeter tube in the manner of Cohen¹ so as to carry on the experiments at a higher temperature and simultaneously with conductivity measurements. To obtain sufficiently clear solutions I found it necessary to filter through a Chamberland candle; I also used for illumination the green ray of the mercury arc. Ring² found that trypsin had no action on the rotation of globulin from horse-serum, unless first acted on by pepsin, in which case there was a diminution by trypsin.

Osmotic Pressure.—I have not yet made sufficient observations to warrant publication of results, but it is to be expected that valuable results will be obtained by regular determination of the depression of the freezing point of solutions under trypsin action.

The Biuret reaction.—The method used for determining this has been already described. Fig. 6 gives the course of the curve of extinction-power compared with the conductivity curve in case of caseinogen. This is from the same experiment as Fig. 1. It will be seen that the biuret reaction has a rapid onset, but that, from the time when the conductivity curve begins to get obviously flatter, the former begins to decline steadily. It is, of course,

¹ Zeitschr. f. physik. Chemie.

² Verh. d. Phys.-Med. Ges. Würzburg. 1902. 35.

well known that the biuret reaction disappears under the action of trypsin, but I do not think that it has been realized that its disappearance commences at so early a stage of the reaction, viz., $2\frac{1}{2}$ hours.

The effect of foreign substances.—In certain experiments on the action of various electrolytes and non-electrolytes on the velocity of reaction, it was noticed that although these various substances in many cases had, in equimolecular concentrations, the same result after some hours, yet the course of the curve was different; some produced a relatively greater effect at the commencement of the reaction. This appears to indicate a selective effect

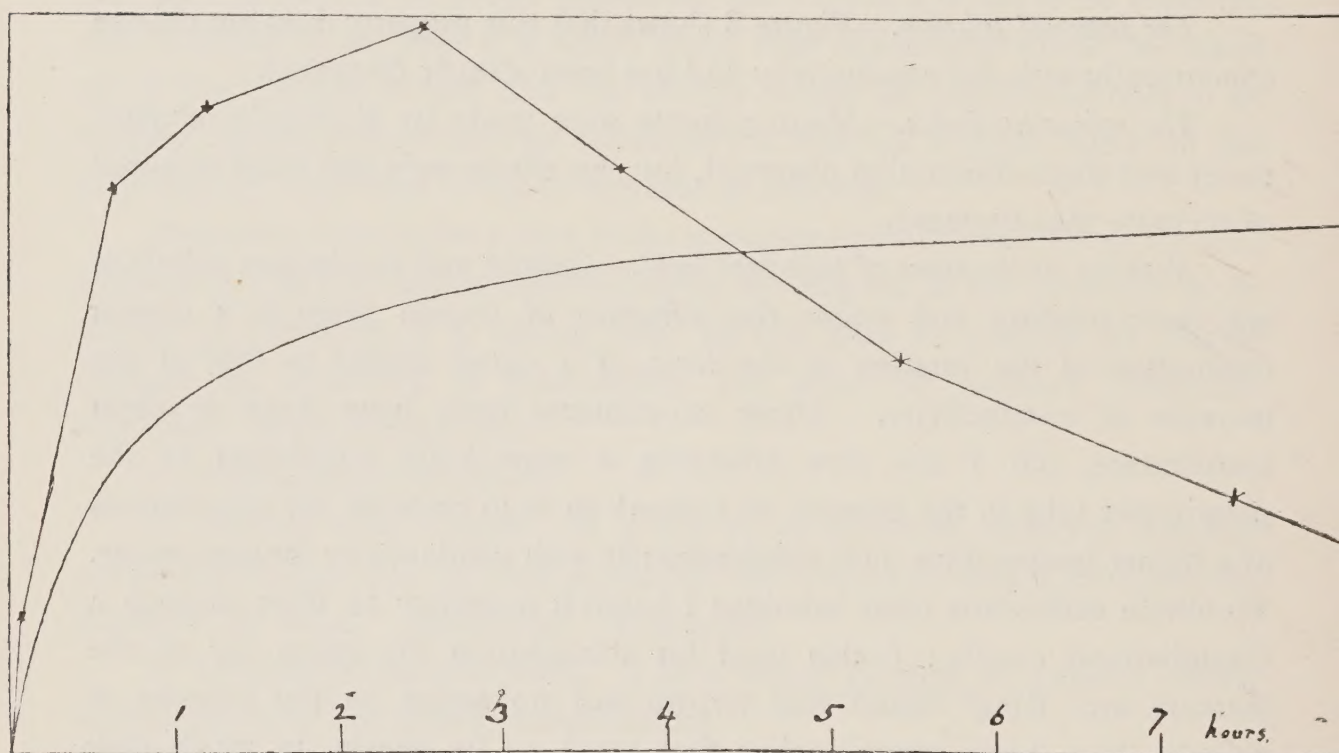


Fig. 6.

on certain of the various concurrent changes going on, but requires further investigation. There are no obvious regularities, as far as the results so far obtained are concerned.

IV. The Mode of Action of Trypsin.

In the discussion of the meaning of the results obtained so far, the mode of treatment adopted by Frankland Armstrong¹ seems to meet the case of trypsin better than any other. I cannot do better than transcribe his explanation almost word for word, merely changing sugar into caseinogen, etc.

¹ Loc. cit., p. 511, etc.

On the assumption that the action of enzymes involves, in the first instance, the combination of enzyme with substrate, which is, in many cases, practically proven "the rate at which the change proceeds will depend on the extent to which the combination of enzyme with substrate takes place as well as on the degree of readiness with which the compound breaks down."

"The proportion of the total substrate present combined with the enzyme and undergoing change at any one moment may be regarded as the *active mass* of the substrate." This is merely a legitimate extension of the conception introduced by Arrhenius¹

"On the hypothesis that the enzyme combines with the substrate, the active mass of the latter will be that portion s of the whole S which is in combination with an amount of enzyme e : it will be convenient to speak of the combination $s + e$ as the *active sys'em.*"

There are four cases to consider, viz.:

"Case I., in which, whatever the amount of substrate present, the quantity of enzyme is relatively small.

Case II., in which there is a difference from Case I., in as much as the quantity of enzyme is relatively considerable.

Case III., in which the amount of enzyme diminishes as the action proceeds.

Case IV., in which the amount of substrate is varied.

Case I.—As action proceeds, since the magnitude of the active system depends on the amount of the enzyme present, it is obvious that, in the initial stages, if the total amount of substrate present S be large compared with s , the enzyme will be in presence of enough substrate molecules to establish the maximum possible number of effective combinations; or, in other words, the magnitude of the active system will remain constant and the change will be expressible, as Brown and Glendinning have pointed out², as a linear function of the time." I find, in fact, that the early stage in some of my curves is very nearly linear, but it is not usually obvious, probably because in most cases the amount of enzyme was relatively great.

"As the action proceeds further, the amount S of substrate present decreases until it is no longer negligible compared with that of the active part s and hence the enzyme will no longer effect the maximum possible number of combinations; the proportion s undergoing change will then be a function of the total mass and the formation of active systems will be governed by the law of mass action. The rate of change will be a logarithmic function of the time."

¹ Zeitschr. f. physik. Chemie. Bd. 4, S. 226.

² Chem. Soc. Trans., 1902, vol. 81, p. 388.

Case II.—"If, on the other hand, the quantity of enzyme used be relatively large, the active mass will be a function of the total mass from the very beginning of the experiment, so that the linear part of the change will escape notice." This case corresponds to most of those which have been investigated by me.

Case III.—"When the amount of the enzyme does not remain constant but for some reasons decreases, the magnitude of the active system will not only be a function of the amount of substrate but also that of the enzyme; it will therefore be represented by an equation of the second order, in which both of two interacting substances decrease—as, for example, is the case in the interaction of an alkali and methyl acetate. Such an expression corresponds to a curve falling off from a logarithmic curve, and therefore giving a series of decreasing values for the velocity constant when this is calculated for the simple logarithmic law. In such a case, the change in its early stages will still be a linear function of the time, as the diminution in the amount of enzyme will not at first materially influence the magnitude of the active system."

This is the case which concerns us most in the treatment of trypsin experiments. Although there is no detectable actual destruction of the enzyme during the first seven or eight hours, a period within which the majority of my experiments were included, we have seen reason to believe that the effect of the products of the reaction is to withdraw trypsin from action by entering into combination with it.

"Stated shortly, the ordinary equation of mass action

$$-\frac{dx}{dt} = H (S-x)$$

where S is the total substrate and x the amount changed in time t , is applicable only to the period during which a constant relatively large proportion of enzyme is present together with a continually decreasing amount of sugar but uninfluenced by the products of change." This period in my experiments is extremely short, since the velocity constant, calculated by the above equation, commences at once to decrease.

"During the final period, when the products of change exercise an influence by withdrawing enzyme from the sphere of action,

$$-\frac{dx}{dt} = K (S-x) (E-y)$$

Where E is the total enzyme, and y the amount withdrawn in combination with the products in time t ."

“During the period, when the proportion of substrate present is very large, x becomes negligible compared with S , so that

$$-\frac{dx}{dt} = KS = k = \text{constant.}”$$

Various enzymes appear to be differently effected by their products, so that the duration of the linear period, according to the last equation, will be of varying duration. Emulsin and maltase are, like trypsin, more affected than are invertase and diastase.

Case IV.—“When the amount of enzyme and water is kept constant whilst that of substrate is increased, it may be supposed that the magnitude of the active system will increase until $s + e$ reaches a maximum, a definite equilibrium being established between enzyme, substrate and water, the whole of the enzyme, perhaps, becoming combined with the substrate. It may be assumed that, if the amount of substrate be further increased, the equilibrium will remain unaffected, notwithstanding that an addition of substrate is practically equivalent to a withdrawal of water.”

“But if $s + e$ remain unaltered, whatever the proportion of substrate present beyond a certain minimum, a constant amount of substrate will undergo change in a given time, although the proportion changed, as also the value of k , will decrease as the concentration is increased.” This is in agreement with the results described in the section of this paper dealing with the effect of increasing the concentration of the substrate, except that beyond a certain point further addition of substrate reduces the velocity, not merely does it remain constant. This is possibly a result of increased internal friction, as pointed out.

We are now in a position to suggest a possible explanation of the fact shown in curve figure 4, that, after a certain period, whatever the amount of enzyme present the change proceeds at the same rate. As the concentration of the substrate diminishes there will come a time when there is only sufficient for a small amount of enzyme to combine with and hydrolyze. If the amount of enzyme present is not very small there will be sufficient of it in all the cases with the varying amounts to act upon all the available substrate. From the period at which the change of conductivity has attained the same value in all, the conditions as regards products of reaction will be the same in all, so that it will be necessary to assume that there is also sufficient excess of enzyme to combine with these products. In those cases in which the curve does not attain the height of that with the greater quantities of enzyme, except after a long period of time, there is probably not sufficient enzyme to satisfy the above requirements.

When we speak of the explanation of enzyme action by combination of enzyme and substrate, we must bear in mind the conditions laid down by Ostwald¹ in order that this shall be a satisfactory explanation. The mere qualitative proof of the existence of an intermediate combination is not, in itself, a proof that the chief reaction is actually accelerated thereby. It should be shown that the sum of all the reactions involved in the formation and decomposition of the intermediate compound in point of fact proceeds more rapidly than the chief reaction. This, as far as I am aware, has only, hitherto, been done in one case, viz., by Brode² who has shown that, in the acceleration by molybdic acid of the reaction between hydrogen peroxide and hydriodic acid, which in itself proceeds at a moderate rate, there is formed a series of permolybdic acids, by the combination of the peroxide and molybdic acid, these are formed rapidly and, when formed, react with great velocity on the hydriodic acid, so that the two reactions with formation of intermediate bodies do actually proceed in sum at a greater rate than the chief reaction.

Since enzymes are colloids, and therefore micro-suspensions, we must also take into consideration the possibility that we have to deal with reactions in a heterogeneous system. The substrate may be taken into the solid enzyme and within it undergo accelerated decomposition, the products of which diffuse out; the process therefore would follow laws of diffusion. Considerations of this kind, suggested by Nernst, have been, to some extent, developed by Herzog³. It does not appear, however, that they can apply to trypsin, since the bodies on which it acts are themselves colloids and therefore unable to penetrate into the colloidal enzyme.

V. Properties of pancreatic juice.

I have at various times taken advantage of the opportunity of using pancreatic juice, as obtained by the injection of secretin, to test certain properties by the application of the conductivity method.

I. Enterokinase.

It has been shown by the present author, in conjunction with E. H. Starling, that enterokinase acts as an enzyme in converting the trypsinogen present in the fresh juice to active trypsin⁴; this is in accordance with the

¹ Zeitschr. f. Electrochemie. Bd. 7, 1000.

² Zeitschr. f. physik. Chemie. Bd. 37, S. 257

³ Zeitschr. f. physiol. Chemie. XLI., p. 416.

⁴ Journal of Physiology. Vol. XXX., p. 61.

original suggestion of Pawlow. It was of interest to see whether there was any change of electrical conductivity to be detected in the reaction. There was in some cases, indeed, a slight diminution of conductivity when enterokinase was allowed to act on secretin-juice at 25° , in other cases a slight increase. It is possible, however, that the diminution may really be of greater amount than appears, since the trypsin as formed acts on the proteids present, producing an increase of conductivity, which would tend to mask the simultaneous process causing diminution. If this diminution be genuine it implies that the process in question is a synthetic one, so that the enzyme, enterokinase, must be one of this class.

An attempt was also made to detect a difference in the initial reaction-velocity when enterokinase, trypsinogen and caseinogen react simultaneously and when caseinogen is acted upon by previously activated juice. It was found, however, that the change of trypsinogen into trypsin proceeds so rapidly at 40° , that only at the extreme initial stage of the reaction was any difference to be detected; it was at this stage distinctly slower in the case of the activation going on concurrently with the action of the trypsin formed. It will be worth while to repeat the experiment at a lower temperature.

2. The proteoclastic enzyme of un-activated juice.

The slow action on fibrin manifested by secretin juice was explained by Starling and myself as due to the presence in it of a small amount of a weak proteoclastic enzyme. I find that this juice has also a slight action on caseinogen, causing an increase of conductivity of about 400 gemmhos in 6 hours. The interesting point about this enzyme is that it is not affected by the anti-body of serum; as already pointed out, in the presence of sufficient serum to annul the action of trypsin on the serum itself, caseinogen present in the same solution is attacked at the usual rate produced by unactivated juice.

The following experiment is also of interest. Starling and I have been able to obtain a serum containing anti-enterokinase¹. This serum was tested as follows: a sufficient quantity was added to a solution of enterokinase to prevent its action on trypsinogen, this mixture was then added to caseinogen solution and pancreatic juice (unactivated). Digestion proceeded at the same rate as in the control vessel containing the same juice without the enterokinase and anti-enterokinase mixture, so that the weak proteoclastic enzyme has no relation to enterokinase.

¹ Journal of Physiology. Vol. XXXII., p. 129., 1905.

VI. Summary of Chief Results.

1. The typical curve of the velocity of trypsin action differs from a logarithmic curve in that it gradually falls away from such a curve, owing to the retarding effect of the products of reaction.

2. The velocity is proportional to the concentration of the substrate up to about 4% in caseinogen; above this and up to about 8% it is independent of the concentration and above 8% it is inversely proportional to it. In no case, however, is the proportionality a linear one, the effect being proportionately less as the concentration rises.

3. If the effect of diminishing concentration as well as that of increasing products of reaction be eliminated, the curve becomes a straight line.

4. There is no evidence of actual destruction of trypsin in presence of proteid solutions up to the seventh or eighth hour at 38°.

5. Trypsin solutions rapidly lose activity when kept, even at 0°.

6. When kept for some time in a warm temperature, trypsin in solution becomes converted into a body resembling a toxoid, which may be called a "zymoid." This body appears to have retained its power of combination with the substrate while becoming comparatively inactive as regards its proteoclastic powers.

7. The chief cause of the increase of electrical conductivity in trypsin digestions is probably the splitting off of the inorganic constituents of the substrate molecules; and also, in the case of caseinogen, to the conversion of organic phosphorus into inorganic phosphates. The change of internal friction has, apparently, very little part in the effect. The behaviour of Curtius' biuret base under trypsin action shows that the production of amino-acids would increase the conductivity to a certain extent.

8. This production of electrolytes is insufficient to account for the retarding action of the products of reaction, they being present in too small a concentration in the total products.

9. There is some evidence that amino-acids are more active as retarding agents than the constituents, such as albumoses and peptones, present as chief constituents of the products of the earlier stage of the reaction.

10. The action of the products is on the enzyme rather than on the substrate; and their mode of action is, most probably, by combining with the enzyme and withdrawing it from the sphere of action. This is supported by the fact that they are at least as active as the substrate in protecting trypsin from destruction by heat.

11. It is shown that in the first stages of the reaction the velocity is in

direct linear proportion to the amount of enzyme present; there follows a stage in which the relation is an exponential function, and finally, if the enzyme is not present in too small an amount, a stage in which the velocity is independent of the concentration of the enzyme.

12. The temperature-coefficient of the reaction-velocity is for 10° between 20° and 30° —5.3, and between 30° and 38° —3.3.

13. Trypsin is capable of acting on caseinogen at a temperature as low as 0° .

14. An anti-trypsin is present in egg-white and serum, which slowly disappears during the reaction with trypsin.

15. There is some evidence of synthetic action on the part of trypsin. The reaction attains a state resembling that of equilibrium, in that it can be changed by alteration of such conditions as are active in this respect in the case of ordinary reversible reactions.

16. The biuret-reaction commences to disappear as early as $2\frac{1}{2}$ hours after the commencement of the action of trypsin on caseinogen.

17. The viscosity of caseinogen solutions undergoes a rapid diminution and then becomes constant, while the conductivity curve continues to rise fairly rapidly.

18. The action is best explained by assuming intermediate combinations between both trypsin and substrate and between trypsin and products of reaction.

19. The existence of a weak proteoclastic enzyme in un-activated pancreatic juice is confirmed.

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